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Application Note

Culture and Characterization of Human iPSC-Derived Pancreatic Duct-Like Organoids

Introduction

Organoids are miniature, simplified versions of an organ that are being increasingly used as in vitro model systems for studying human organ development and tissue regeneration, modeling diseases, screening drugs, and investigating personalized medicine. Organoids are derived from pluripotent or adult tissue-specific stem cells, which are driven to form 3D structures when cultured in the presence of a cell scaffolding matrix and growth factors or small molecules that promote their differentiation into specific cell lineages. The resulting "mini organs" consist of multiple different cell types that self-organize to mimic the structure of the organ they are modeling and exhibit functional similarities. Organoid models have been developed for a wide variety of different organs, including brain, intestine, kidney, liver, lung, stomach, and pancreas, among others.

In this study, we demonstrate the ability to culture and characterize human induced pluripotent stem cell (iPSC)-derived pancreatic duct-like organoids (PDLOs). This type of organoid model system is of great interest as it is being used to study a variety of diseases, including cystic fibrosis, pancreatitis, and pancreatic ductal adenocarcinoma (PDAC), which is a particularly aggressive form of cancer with a 5-year survival rate of only 5%. PDLOs allow researchers to study the development and progression of these diseases, and they provide a platform for screening and validating potential new drugs or predicting their effects.

Using a protocol adapted from Breunig, *et al.* (2021), iPSCs were differentiated with R&D Systems™ proteins and small molecules into functional PDLOs. A high differentiation efficiency was achieved, as indicated by the expression of target cell markers in the intermediary and final cell populations. At the end of the 30-day differentiation protocol, 95% of the cells expressed the pancreatic ductal cell marker, KRT19, and 40% expressed SOX9, a transcription factor required for pancreatic ductal development. Immunofluorescent staining of the PDLOs showed the expression of these ductal cell markers in the expected locations and demonstrated the high quality of the organoid structures. Further characterization using a CFTR assay showed that the PDLOs had functional CFTR ion channels and treatment with CFTR inhibitors suggested that these organoids could serve as an effective drug screening platform for identifying new treatments for pancreatic diseases.

Key Takeaways

- R&D Systems' reagents and instruments can be used to support the culture, characterization, and functional analysis of human iPSC-derived pancreatic duct-like organoids (PDLOs).
- Flow cytometry analysis demonstrated that the PDLO differentiation was highly efficient, with 95% of the cells expressing KRT19 and 40% expressing SOX9, two characteristic markers of pancreatic ductal cells.
- Orthogonal validation on the Leo™ System powered by Simple Western™ technology confirmed that expression of the NKX6.1 and PDX-1 progenitor markers decreased during differentiation, while the mature ductal cell markers, SOX9, KRT19, and E-Cadherin were expressed at high levels in the PDLOs.
- Immunofluorescent staining showed that the ductal cell markers were expressed in the expected locations and demonstrated the high quality of the organoid structures.
- The PDLOs were shown to have functional CFTR ion channels and treatment with CFTR inhibitors demonstrated that these organoids could serve as an effective drug screening platform for testing the efficacy of drugs aimed at treating pancreatic diseases.

Materials

R&D Systems Products for iPSC Maintenance and Expansion	Catalog #
ExCellerate™ iPSC Expansion Medium, Animal-Free, GMP	CCM036-GMP
Cultrex™ UltiMatrix Reduced Growth Factor Basement Membrane Extract	BME001-05
Y-27632 dihydrochloride (ROCK inhibitor)	1254
Recombinant Human FGF basic Heat Stable Protein	BT-FGFBHS

R&D Systems Products for Pancreatic Duct-Like Organoid Differentiation	Catalog #
Cultrex UltiMatrix Reduced Growth Factor Basement Membrane Extract	BME001-05
Y-27632 dihydrochloride (ROCK inhibitor)	1254
L-Ascorbic acid	4055
Recombinant Human Activin A	11348-AC
CHIR 99021	4423
Recombinant Human FGF basic Heat Stable Protein	BT-FGFBHS
Recombinant Human FGF-10	345-FG
Dorsomorphin	3093
Recombinant Human Wnt-3a	5036-WN
LDN 193189	6053
SANT-1	1974
Retinoic acid	0695
Nicotinamide	4106
Recombinant Human EGF	236-EG
Recombinant Human KGF/FGF-7	BT-KGF

R&D Systems Products for Cell Lineage Characterization	Catalog #
Alexa Fluor® 488-conjugated Mouse Anti-Human CXCR4 Monoclonal Antibody	FAB173G
APC-conjugated Mouse Anti-Human CD117/c-kit Monoclonal Antibody	FAB332A
PE-conjugated Mouse Anti-Human/Mouse PDX-1/IPF1 Monoclonal Antibody	IC2419P
NorthernLights™ NL557-conjugated Goat Anti-Human SOX9 Polyclonal Antigen Affinity-purified Polyclonal Antibody	NL3075R
Alexa Fluor 488-conjugated Sheep Anti-Human Cytokeratin 19 (KRT19) Antigen Affinity-purified Polyclonal Antibody	IC3506G
Rat Anti-Human Claudin-1 Monoclonal Antibody	MAB4618
Mouse Anti-Human Cytokeratin 19 Monoclonal Antibody	MAB35061
Mouse Anti-Human SOX9 Monoclonal Antibody	MAB3075
NorthernLights NL493-conjugated Donkey Anti-Mouse IgG Antigen Affinity-purified Polyclonal Antibody	NL009
NorthernLights NL557-conjugated Goat Anti-Rat IgG Antigen Affinity-purified Polyclonal Antibody	NL013

Bio-Techne, R&D Systems, and Novus Biologicals Products for Characterization of Pancreatic Duct-Like Organoid Markers by Simple Western Technology	Catalog #
Leo System	004-550
Leo 12-230 kDa Separation Module	SWSM-W014
EZ Standard Pack 1	PS-ST01EZ-8
Anti-Rabbit Detection Module	DM-001
Anti-Mouse Detection Module	DM-002
Total Protein Detection Module	SWDM-TP21
RePlex™ Module	RP-001
Goat Anti-Human/Mouse NKX6.1 Antigen Affinity-purified Polyclonal Antibody	AF5857
Goat Anti-Human PDX-1/IPF1 Antigen Affinity-purified Polyclonal Antibody	AF2419
Mouse Anti-Human Cytokeratin 19 Monoclonal Antibody	NBP2-15186
Rabbit Anti-Human/Mouse SOX9 Antigen Affinity-purified Polyclonal Antibody	NBP1-85551
Mouse Anti-Human E-Cadherin Monoclonal Antibody	MAB1838

R&D Systems Products for Functional Characterization of Pancreatic Duct-Like Organoids	Catalog #
Forskolin	1099
GlyH 101	5485
$CFTR_{inh}$ 172	3430

Experimental Workflow

A protocol adapted from Breunig, *et al.* (2021) was used to generate pancreatic duct-like organoids (PDLOs) from human induced pluripotent stem cells (iPSCs).[1] Briefly, iPSCs were seeded in a 24-well plate and allowed to reach 75-95% confluency before the differentiation protocol was started (Figure 1). The differentiation was performed in 2D culture through the pancreatic progenitor (PP) stage, and then the cells were reseeded for 3D culture using Cultrex UltiMatrix RGF Basement Membrane Extract and the other reagents listed to promote pancreatic duct-like organoid differentiation. The PDLOs obtained from this protocol could be further expanded beyond Day 30 depending on the downstream applications.

Throughout the protocol, brightfield microscopy and flow cytometry were used to characterize the cell morphologies and phenotypes at defined stages of development, including definitive endoderm (DE), pancreatic progenitor (PP), and the final PDLOs that were obtained at the end of the 30-day differentiation. Simple Western analysis was used to compare the expression of the progenitor markers, NKX6.1 and PDX-1 in the pancreatic progenitor cell population and the PDLOs. The PDLOs were further characterized by immunofluorescent imaging and Simple Western analysis to assess the expression of mature ductal cell markers, including SOX9, KRT19, and E-Cadherin. The functionality of the PDLOs was evaluated using a cystic fibrosis transmembrane conductance regulator (CFTR) activity assay. CFTR activation was induced with forskolin and the CFTR inhibitors, GlyH 101 or $CFTR_{inh}$ 172, were used to block forskolin-induced PDLO swelling.

FIGURE // 1

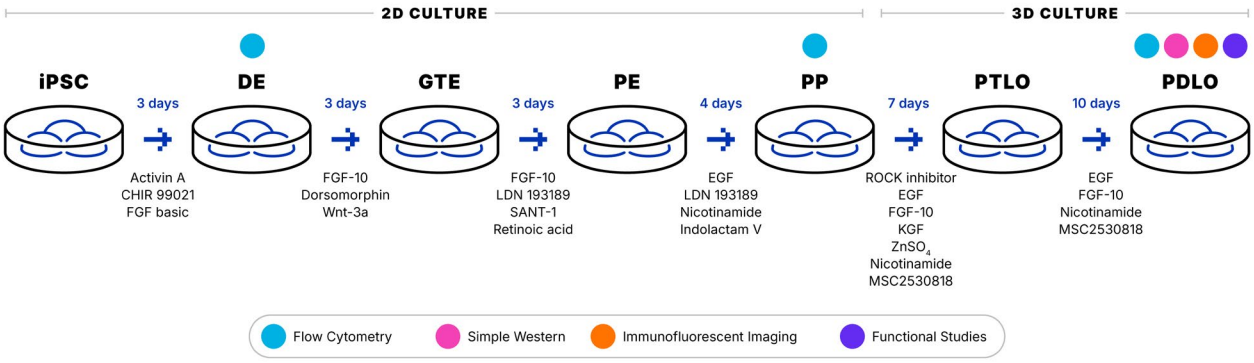
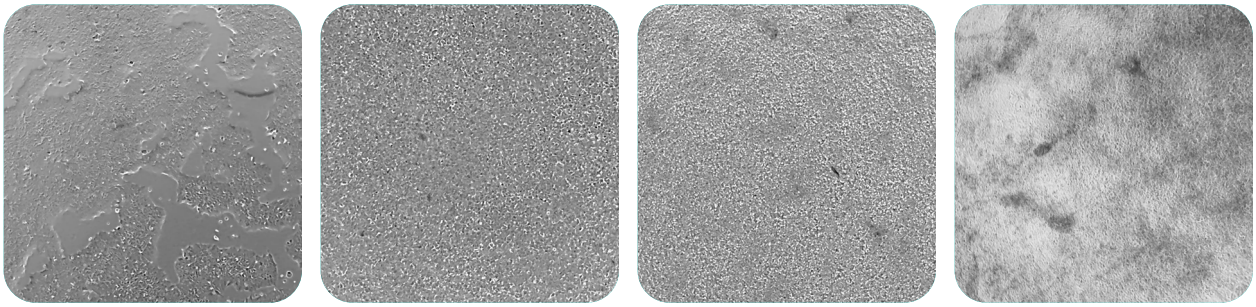


Figure 1. Differentiation Workflow Used to Generate Human iPSC-Derived Pancreatic Duct-Like Organoids. The workflow was adapted from a protocol described in Breunig *et al.*, where a human induced pluripotent stem cell (iPSC) line was differentiated into pancreatic duct-like organoids (PDLO). The 30-day differentiation encompasses 5 major intermediate stages: 1) definitive endoderm (DE); 2) gut tube endoderm (GTE); 3) pancreatic endoderm (PE); 4) pancreatic progenitors (PP); and 5) pancreatic trunk-like organoids (PTLO). The source and catalog numbers for the R&D Systems reagents used for the differentiation protocol are listed in the Materials section. Throughout the workflow, the defined stages of development and the final PDLOs were characterized using a variety of techniques, including brightfield microscopy, flow cytometry, immunofluorescent imaging, Simple Western, and functional assays.

Results

The protocol shown in Figure 1 was used to generate pancreatic duct-like organoids (PDLOs) from human iPSCs.[1] Throughout the 30-day differentiation, brightfield microscopy and flow cytometry were used to characterize the intermediary stages of cell differentiation. As shown in the data, the cells displayed the expected morphology (Figure 2) and expressed high levels of the expected markers (Figure 3) at each of the key differentiation steps. A high differentiation efficiency was achieved as ≥ 90% of the cells in the definitive endoderm cell population co-expressed CXCR4 and CD117/c-kit; ≥ 80% of the pancreatic progenitor cell population co-expressed PDX-1 and NKX6.1 (Figure 3A); and in the final cell population, 95% of the cells stained positive for KRT19, a common marker expressed both on the cell membrane and in the cytoplasm of pancreatic ductal cells, and 40% stained positive for SOX9, a transcription factor essential for pancreatic ductal development (Figure 3B).

FIGURE 2 // 2D CULTURE



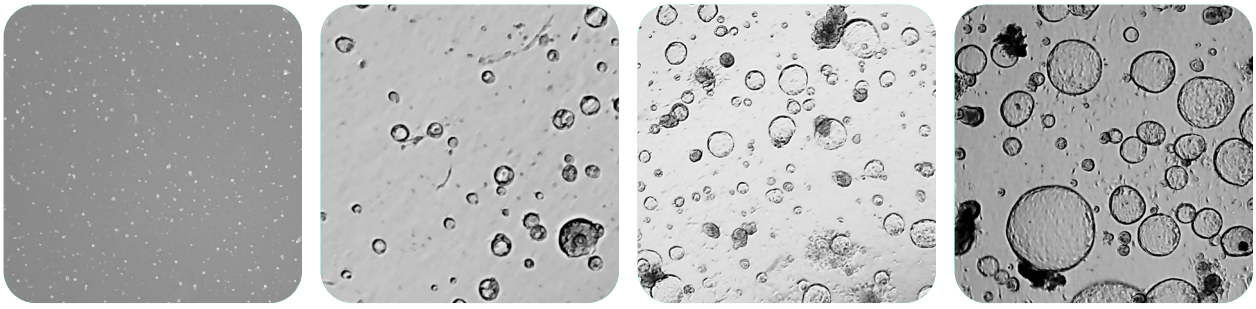
iPSC
(Day 0)

Definitive Endoderm
(Day 2)

Gut Tube Endoderm
(Day 5)

Pancreatic Progenitors
(Day 12)

3D CULTURE



PP Seeded in UltiMatrix
(Day 13)

PDLO
(Day 24)

PDLO
(Day 31)

PDLO
(Day 34)

Figure 2. Cell Morphologies at Each Stage of PDLO Differentiation. Representative brightfield images of cells taken during the 30-day PDLO differentiation workflow. The differentiation was performed in 2D culture through the pancreatic progenitor (PP) stage. For 3D culture, the pancreatic progenitor cells were reseeded as single cells in Cultrex UltiMatrix RGF BME (R&D Systems, Catalog # BME001-05) using either a "sandwich" or "dome" method as described in Breuning, *et al.* (2021). After two weeks of 3D culture, the pancreatic progenitor cells had differentiated into pancreatic duct-like organoids, resembling functional human pancreatic ducts. Images are shown at 4x magnification.

FIGURE // 3

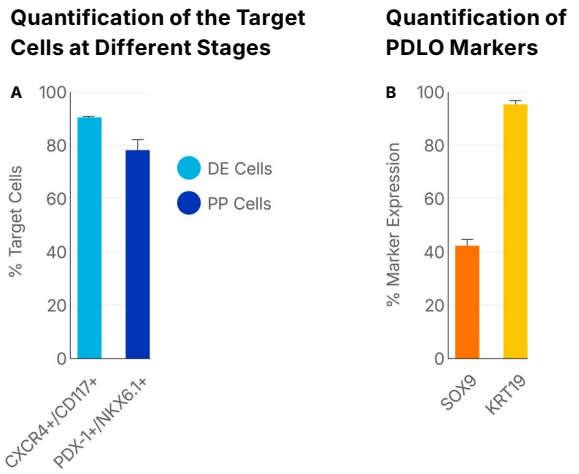


Figure 3. Characterization of Cellular Markers at Key Stages of PDLO Differentiation Using Flow Cytometry. (A) iPSC-derived definitive endoderm (DE) cells and pancreatic progenitor (PP) cells were stained for markers of the expected cell phenotype and analyzed by flow cytometry. Antibodies used include an Alexa Fluor® 488-conjugated Anti-Human CXCR4 Antibody (R&D Systems, Catalog # FAB173G), an APC-conjugated Anti-Human CD117/c-kit Antibody (R&D Systems, Catalog # FAB332A), a PE-conjugated Anti-Human/Mouse PDX-1/IPF1 Antibody (R&D Systems, Catalog # IC2419P), and an Alexa Fluor 647-conjugated anti-human NKX6.1 antibody. (B) After differentiation, the iPSC-derived pancreatic duct-like organoid (PDLO) cells were stained with the NorthernLights NL557-conjugated Anti-Human SOX9 Polyclonal Antibody (R&D Systems, Catalog # NL3075R) and an Alexa Fluor 488-conjugated Anti-Human KRT19 Polyclonal Antibody (R&D Systems, Catalog # IC3506G). The data is represented as the average of technical replicates +/- SD.

PDLO differentiation was further confirmed by comparing the expression of the progenitor markers, NKX6.1 and PDX-1 in lysates collected from day 13 pancreatic progenitor cells and day 28 PDLOs. The expression levels were determined using Simple Western analysis on the Leo System, which is a high-throughput, fully automated, capillary-based immunoassay platform that enables fast, simple, and sensitive protein detection and quantification.

As expected, the Simple Western data demonstrated that the expression of the NKX6.1 and PDX-1 progenitor markers decreased during differentiation, indicating a commitment to the ductal cell lineage (Figure 4). Further characterization of the PDLOs by Simple Western showed that the organoids expressed high levels of the mature ductal cell markers, SOX9, KRT19, and E-Cadherin (Figure 5), reinforcing the flow cytometry data shown in Figure 3B.

FIGURE // 4

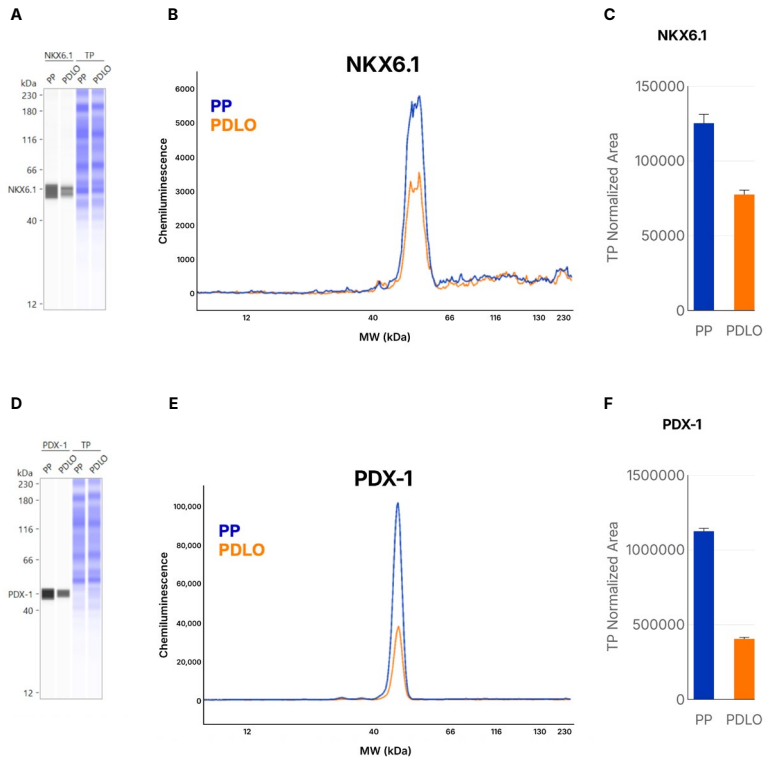


Figure 4. Simple Western Analysis of the Progenitor Markers, NKX6.1 and PDX-1 in Pancreatic Progenitor Cells and PDLOs. Simple Western analysis with the automated Leo System (Bio-Techne, Catalog # 004-550) was used to compare the expression of the pancreatic progenitor markers, NKX6.1 and PDX-1 in cell lysates prepared from pancreatic progenitor (PP) cells collected at day 13 and pancreatic duct-like organoids (PDLOs) collected at day 28 of the differentiation protocol. Antibodies used include an Anti-Human/Mouse NKX6.1 Polyclonal Antibody (R&D Systems, Catalog # AF5857) and an Anti-Human PDX-1/IPF1 Polyclonal Antibody (R&D Systems, Catalog # AF2419). Protein of interest and total protein (TP) expression were detected in the same capillary using RePlex with chemiluminescence detection. (A) and (D) Representative lane view of NKX6.1 and PDX-1 detection, respectively, alongside total protein expression; (B) and (E) Representative electropherograms of NKX6.1 and PDX-1 detection, respectively; (C) and (F) Measurement of NKX6.1 and PDX-1 expression, respectively (n=3). The experiment was performed using 1 mg/mL of lysate and 8 µg/mL of primary antibody.

FIGURE // 5

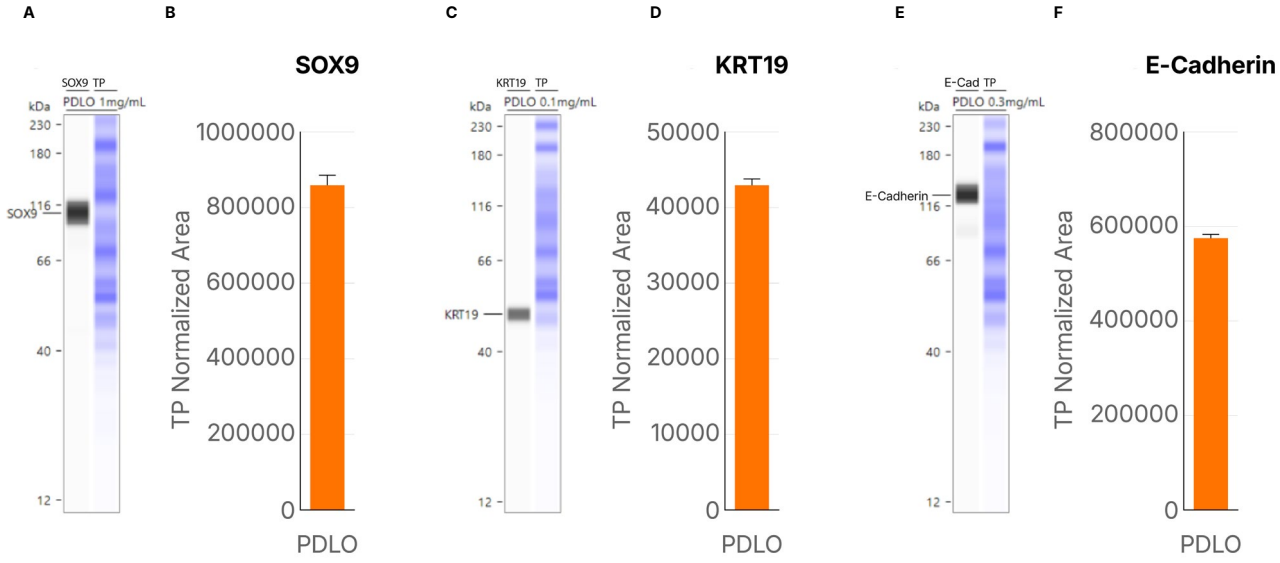


Figure 5. Detection of PDLO Markers by Simple Western. Simple Western analysis with the automated Leo System (Bio-Techne, Catalog # 004-550) was used to assess the expression of mature ductal cell markers in cell lysates prepared from pancreatic duct-like organoids (PDLOs) collected at day 28 of the differentiation protocol. Antibodies used include an Anti-Human/Mouse SOX9 Polyclonal Antibody (Novus Biologicals, Catalog # NBP1-85551), an Anti-Human Cytokeratin 19 (KRT19) Monoclonal Antibody (Novus Biologicals, Catalog # NBP2-15186), and an Anti-Human E-Cadherin Monoclonal Antibody (R&D Systems, Catalog # MAB1838). Protein of interest and total protein (TP) expression were detected in the same capillary using RePlex with chemiluminescence detection. (A), (C), and (E) Representative lane view of SOX9, Cytokeratin 19 (KRT19), and E-Cadherin detection, respectively, alongside total protein expression. (B), (D), and (F) Measurement of SOX9, KRT19, and E-Cadherin expression, respectively (n=3). The experiment was performed using 1 mg/mL of lysate and 3.2 µg/mL of primary antibody for SOX9 detection; 0.1 mg/mL of lysate and 8 µg/mL of primary antibody for KRT19 detection; and 0.3 mg/mL of lysate and 20 µg/mL of primary antibody for E-Cadherin detection.

To visually confirm the successful differentiation of pancreatic duct-like organoids, immunofluorescent staining was performed to assess the expression and localization of SOX9 and KRT19 (Figure 6). Additionally, staining of Claudin-1, which is expressed on the membrane of pancreatic ductal epithelial cells and localized to the tight junctions between adjacent cells, was used to evaluate the cellular architecture and verify that the organoids successfully formed a polarized epithelial layer. The staining results confirmed that each of the markers was expressed in the expected locations in the PDLOs and demonstrated the high quality of the organoid structures (Figure 6).

FIGURE // 6

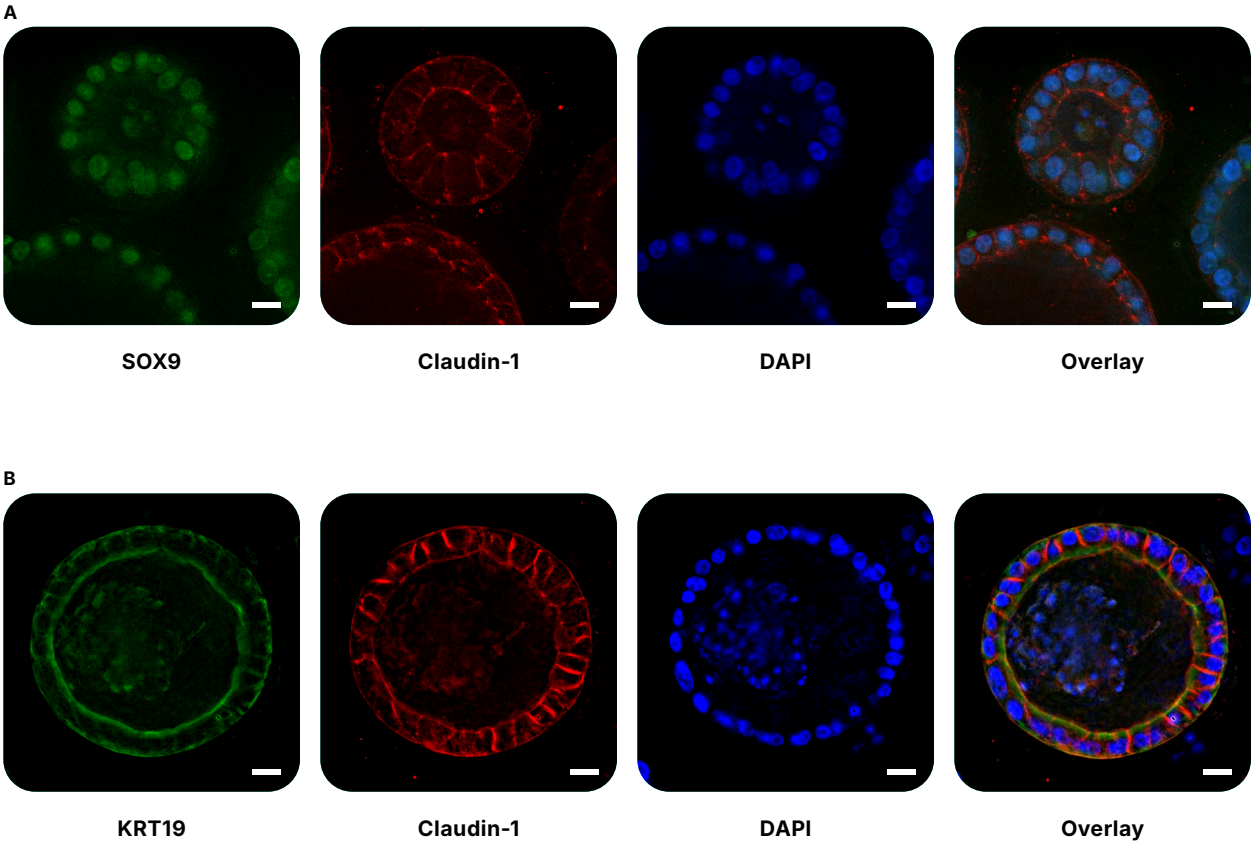


Figure 6. Immunofluorescent Staining of iPSC-Derived Pancreatic Duct-Like Organoids. Representative images of cells stained for characteristic markers of pancreatic ductal cells and analysis of organoid structures. iPSC-derived pancreatic duct-like organoids were fixed and stained with (A) an Anti-Human SOX9 Monoclonal Antibody (R&D Systems, Catalog # MAB3075) and an Anti-Human Claudin-1 Monoclonal Antibody (R&D Systems, Catalog # MAB4618) or (B) an Anti-Human KRT19 Monoclonal Antibody (R&D Systems, Catalog # MAB35061) and an Anti-Human Claudin-1 Monoclonal Antibody (R&D Systems, Catalog # MAB4618), followed by secondary antibody staining with a NorthernLights™ 493-conjugated Anti-Mouse IgG Polyclonal Antibody (R&D Systems, Catalog # NL009; green) and a NorthernLights™ 557-conjugated Anti-Rat IgG Polyclonal Antibody (R&D Systems, Catalog # NL013; red). Cell nuclei were stained with DAPI (R&D Systems, Catalog # 5748; blue) and the images were overlaid. Scale bar: 200 µm.

Following assessment of the PDLO markers, functionality of the organoids was evaluated using a cystic fibrosis transmembrane conductance regulator (CFTR) activity assay. CFTR is a protein that plays a significant role in pancreatic ductal function as it facilitates the movement of chloride and bicarbonate ions across the apical membrane. Secretion of bicarbonate ion is essential for neutralizing the acidic digestive enzymes released by the pancreatic acinar cells. In functional PDLOs, CFTR activation, which is typically stimulated by a forskolin-induced increase in intracellular cAMP levels, causes an efflux of chloride into the lumen of the organoid. This creates an osmotic gradient that leads to an influx of water into the ductal lumen, causing the PDLOs to swell.

When this assay was performed on the PDLOs generated in this study, forskolin-induced swelling of the PDLOs was observed, demonstrating that the organoids have functional CFTR channels (Figure 7). This observation was confirmed by adding the CFTR inhibitors, GlyH 101 or $CFTR_{inh}$ 172 together with forskolin, which effectively blocked the forskolin-induced PDLO swelling. Together, these results show that the PDLOs had normal CFTR function, and their response to the CFTR inhibitors suggests that the PDLOs could be used as a model system to test the efficacy or toxicity of drugs aimed at improving or restoring CFTR function.

FIGURE // 7

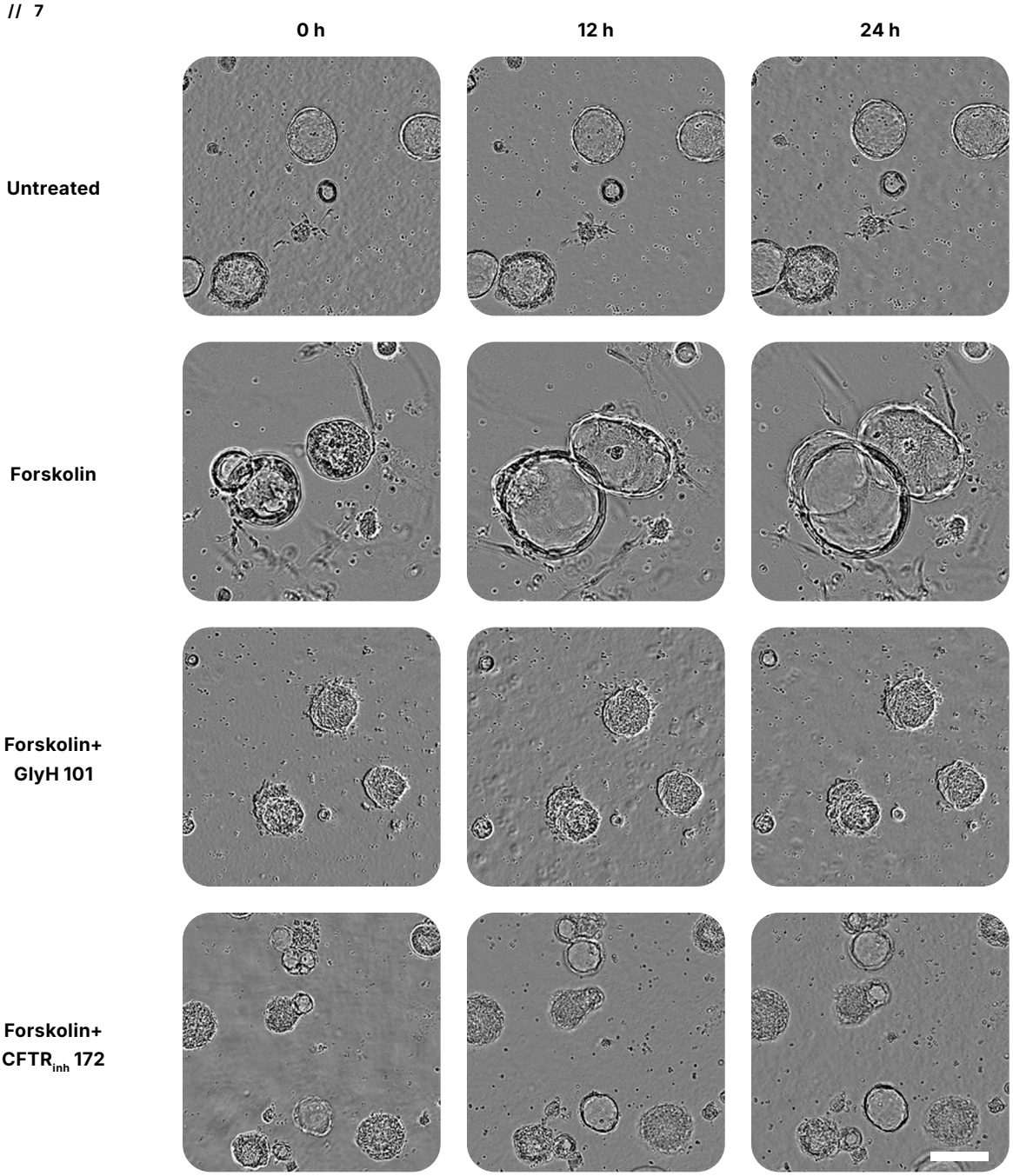


Figure 7. CFTR Assay for the Functional Assessment of iPSC-Derived Pancreatic Duct-Like Organoids. Representative images of pancreatic duct-like organoids (PDLOs) treated with forskolin +/- CFTR inhibitors. The PDLOs seeded in Cultrex UltiMatrix RGF Basement Membrane Extract (R&D Systems, Catalog # BME001-05) were treated with 20 mM Forskolin (R&D Systems, Catalog # 1099) for 0, 12, or 24 hours, and swelling of the PDLOs was observed. When the PDLOs were treated with both forskolin and the CFTR inhibitors, GlyH 101 (20 mM; R&D Systems, Catalog # 5485) or $CFTR_{inh}$ 172 (10 mM; R&D Systems, Catalog # 3430) for similar amounts of time, the forskolin-induced swelling of the PDLOs was blocked. Scale bar: 200 µm.

Conclusions

This study demonstrates the successful use of R&D Systems reagents and instruments for the culture, differentiation, and characterization of human iPSC-derived pancreatic duct-like organoids (PDLOs). Differentiation of the PDLOs was highly efficient, with 95% of the cells in the final PDLO cell population expressing the characteristic pancreatic ductal cell marker, KRT19, and 40% expressing the SOX9 transcription factor required for pancreatic ductal development. Orthogonal validation by Simple Western analysis confirmed the high level expression of mature ductal cell markers in the PDLOs.

Immunofluorescent staining showed the expression of KRT19, SOX9, and Claudin-1 in the expected locations and demonstrated the high quality of the PDLO structures. A CFTR assay indicated that the PDLOs had functional CFTR ion channels, and their response to CFTR inhibitors suggested they could serve as an effective drug screening platform. Based on these results, we conclude that R&D Systems reagents can be used to efficiently generate a pancreatic duct-like organoid model system for investigating the pathology of pancreatic diseases, evaluating new drug treatments, and enabling the advancement of cell and gene therapy research.

REFERENCES

1. Breunig, M. *et al.* (2021) STAR Protoc. **2**:100913.

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